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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A01N 1/02, C12N 5/00 // A61K 35/54, 35/52, A61D 19/00	A1	(11) International Publication Number: WO 92/21234 (43) International Publication Date: 10 December 1992 (10.12.92)
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(54) Title: COMPOSITION AND METHOD FOR CULTURING AND FREEZING CELLS AND TISSUES (57) Abstract The present invention includes a composition and method for culturing and freezing cells and tissues without serum or serum products in the culture medium by substituting hyaluronic acid for the serum or serum products. The composition and method are especially well-suited for culturing and freezing embryos, ova and sperm. The present invention reduces the risk of transmittal of microorganisms such as viruses.		

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COMPOSITION AND METHOD FOR CULTURING AND FREEZING CELLS AND TISSUES

Field of Invention

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This invention relates in general to a composition and method for culturing and freezing cells and tissues and more particularly relates to a composition and method for harvesting, culturing and freezing embryos using a medium containing hyaluronic acid.

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Background of the Invention

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The term "serum" as used herein means the liquid portion of the blood that remains after blood cells and fibrinogen/fibrin are removed. The term "plasma" as used herein means the liquid portion of blood that remains after blood cells are removed. The term "serum products" means any component of the serum, such as soluble proteins, lipids or carbohydrates or combinations thereof. An example of a serum protein is albumin.

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The use of freezing techniques to freeze various cells and tissues for long term storage has become increasingly popular. In particular, the agricultural industry has successfully utilized these freezing techniques in animal husbandry. Furthermore, freezing techniques have been used to preserve ova, sperm and embryos from humans for later use.

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A large industry is developing that is concerned with methods of fertilizing and transferring ova and embryos to surrogate females. The advantages of this technology to the animal husbandry industry include increasing the reproductive rate of valuable animals, decreasing the generation interval, increasing the number of progeny per female through controlled multiple births, and transporting embryos with selected genetic characteristics to distant places. This technology has been used for over 300 species including, but not limited to, cattle, swine, sheep, goats and other agriculture animals. The technology has also been used for freezing human embryos.

By way of example, a brief description of the biology of bovine reproduction follows: Bovine embryos move from the oviduct to the uterus four to five days after estrus (three to four days after ovulation), although in super ovulated cows, a few remain in the oviduct through day seven. A high percentage of embryos can usually be recovered nonsurgically from the uterus six or more days after the beginning of estrus.

Several procedures are currently available for recovering embryos. One such procedure comprises inserting a Rusch or Foley catheter through the cervix into the uterus by palpating through the wall of the rectum with one hand as is done for artificial insemination. The latex catheter usually consists of three channels for inflow, outflow, and inflation of the balloon-like cuff that prevents the escape of fluid after insertion. Each uterine horn is filled and emptied several times with 30-200 ml of fluid each time according to the size of the uterus. The embryos are flushed out with this fluid into large graduated cylinders. Embryos can be filtered with a 50-70 μ mesh filter or allowed to settle for thirty minutes and can then be located under a stereo microscope by searching through an aliquot from the bottom of the cylinder. They are

then stored in small containers or loaded into 0.25 to 5 ml straws until transferred.

In addition, the embryos can be recovered surgically. This is the procedure of choice for laboratory animals and for certain agricultural animals and may be the method of individual operator preference for large agricultural animals.

Embryos from the one cell to the early blastocyst stage (7 to 8 days after estrus) are between 120 and 140 micrometers in diameter exclusive of the zone of pellucida. Between days 8 and 10, they double in diameter, hatch from the zone of pellucida, and then grow to 20 cm or more in length by day 18. Since bovine embryos form no intimate attachment to the uterus before day 18, they can be recovered nonsurgically until this time, although they are increasingly prone to damage after day 14. It appears that a larger number of normal embryos can be obtained nonsurgically 6 to 8 days after estrus than at other times. (See U.S. Patent No. 4,780,451.)

After the embryos are collected from the donor cow, they should be transferred to recipient animals within a short period of time. Alternatively, the embryos can be cooled to refrigerator temperatures or frozen. Conventional freezing media contain some type of serum. For example, newborn calf serum or bovine serum albumin (BSA) plus glycerol and/or other constituents have been widely used in media for embryo freezing as both cryoprotectants and surfactants.

However, these sera have a major problem in that they can transfer viral contaminants to the embryo including, but not limited to, bovine virus diarrhea (BVD), infectious bovine rhinotrachitis (IBR), bovine spongiform encephalopathy (BSE), Scrapie, blue tongue, and foot and mouth disease. In addition, serum from animals inherently

adds undefined components to the freezing media allowing for the possibility of deleterious effects on the embryos.

5 There is accordingly a need for a composition and method for culturing and freezing living tissues and cells, including embryos, which do not contain serum or serum products from animals. There is also a need for a culturing and freezing medium which is completely defined and does not contain any serum or serum products from animals or humans. The freezing medium should protect the living tissue or cells that are being frozen and allow high viability of the cells or tissue when they are thawed.

Summary of the Invention

15 The present invention comprises a composition and method for culturing and freezing cells or tissues comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products. Various types of cells or tissues that could be frozen using a conventional freezing medium containing hyaluronic acid include, but are not limited to, embryos, unfertilized ova or sperm.

20 The present invention also includes flushing media for flushing or washing ova or embryos from the reproductive tract of a female. The medium contains hyaluronic acid in place of serum or other serum products. In addition, the present invention includes growth media which contains hyaluronic acid. This growth medium can be used to allow the embryo to grow before it is transferred or frozen.

25 The preferred molecular weight of the hyaluronic acid contained in the freezing medium is between approximately 1.0×10^4 and 3×10^6 Daltons. The more preferred molecular weight of the hyaluronic acid is between approximately 5×10^5 to 1.2×10^6 Daltons. The most

preferred molecular weight of the hyaluronic acid as used in the present invention is 7.5×10^5 Daltons.

5 The concentration of hyaluronic acid in the culture and freezing media of the present invention is between approximately .01 and 10 mg/ml. A preferred concentration of hyaluronic acid is between approximately 0.05 and 5 mg/ml with a more preferred concentration being between approximately 0.5 mg/ml to 2 mg/ml. A most preferred concentration of hyaluronic acid is approximately 1.0 mg/ml.

10 Accordingly, it is an object of the present invention to provide an effective composition and method for culturing and freezing cells and tissues and for maintaining their viability after thawing.

15 It is another object of the present invention to provide a medium for flushing ova or embryos from a female.

It is another object of the present invention to provide a culture and freezing medium that will reduce or eliminate the transfer of microorganisms, such as viruses, to the cells and tissues during the freezing process.

20 It is another object of the present invention to provide a medium that does not contain serum albumin.

It is yet another object of the present invention to provide a medium for freezing embryos, ova and sperm.

25 It is yet another object of the present invention to provide a medium that is completely defined for freezing living cells and tissues.

It is yet another object of the present invention to avoid deleterious effects on living cells and tissues being frozen.

30 It is yet another object of the present invention to increase the reproductive capability of female cows.

It is a further object of the present invention to produce superior agriculture animals, including but not limited to, cattle, swine, horses, sheep, deer and goats.

It is a further object of the present invention to produce superior laboratory animals including, but not limited to, rats, mice, guinea pigs and rabbits.

5 It is a further object of the present invention to provide a flushing, culturing and freezing medium for culturing and freezing human ova, sperm and embryos that does not contain serum, serum albumin or other blood products including plasma.

10 It is yet another object of the present invention to provide a culture medium capable of extending the useful life of embryos *in vitro*.

It is another object of the present invention to provide a medium for the culturing and freezing of human embryos, ova and sperm.

15 Other objects and advantages of the present invention will become more readily apparent from the following description of a preferred embodiment.

Detailed Description of Preferred Embodiments

20 The present invention includes a composition for culturing and freezing cells or tissues comprising a solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products.

25 The present invention also includes a method for culturing cells or tissues comprising the step of mixing the cells or tissues with a medium, the medium comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a
30 concentration effective to be a substitute for serum or serum products.

The present invention also includes a method for
35 freezing cells or tissues comprising the steps of (a) mixing the cells or tissues with a medium, the medium comprising a culture solution containing the nutritional substances necessary

to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products; and (b) lowering the temperature of the culture medium sufficiently to preserve the embryos in a viable condition.

The present invention further includes a method for flushing ova or embryos from a donor female comprising the step of flushing the reproductive tract, oviduct or uterus, of an appropriate female with a medium, the medium comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products

The hyaluronic acid is a substitute for serum or serum products. The culture and freezing medium may be any conventional medium, such as Dulbecco's phosphate buffered saline or Ham's F-10 medium. According to the present invention, hyaluronic acid can replace serum in conventional culture and freezing media thereby reducing or eliminating the risk of transfer of harmful microorganisms such as viruses which may be present in the serum.

The basic culture media are well known to those of ordinary skill in the art. These media include a solution containing substances which are osmotically compatible with the cells and/or tissues which are placed in the media and, for long term culture, these media also contain substances which are nutritionally necessary to support the cells and/or tissues.

The present invention is particularly useful in the culturing and freezing of embryos. The media which comprise the present invention may either be used simply as a collection material to flush the embryo from the reproductive tract of an animal and to hold the embryo after it has been flushed from the donor animal or they may be used to preserve the embryo for an extended period of time. If the culture medium of the present invention is used for an

extended period of time, it may be frozen with the embryo by the addition of a cryopreservation agent, including but not limited to, glycerol or dimethyl sulfoxide. In addition, the media which comprise the present invention may be used to culture the embryo to allow it to grow before transfer to the recipient female. It is to be understood that the present invention can be used equally well for both animal embryos or human embryos, as well as for ova and sperm.

Hyaluronic acid occurs in vitreous humor, synovial fluid (lubricating fluid of the joints), skin, umbilical cord, hemolytic streptococci Type A and C, pathologic joints and rooster combs. It is part of the gel-like ground substance of connective tissues and serves as a shock absorbent and lubricant. It occurs both free as the anion with simple cations or it can be associated with protein cations in a variety of tissues. In these tissues, the compound acts as a cement and a permeability barrier by allowing passage of metabolites but blocking invasion by bacteria.

Heretofore, hyaluronic acid has been used in many therapeutic applications. U.S. Patent No. 4,141,931 to Balazs discloses the replacement of vitreous humor with hyaluronic acid during ophthalmic surgical procedures. Hyaluronic acid is also used as a antimicrobial agent (U.S. Patent No. 4,784,991 to Nimrod); in cosmetic preparations (U.S. Patent No. 4,187,291 to Marissal and U.S. Patent No. 4,303,676 to Balazs); as a treatment for anesthesia (U.S. Patent No. 3,898,329 to Revia); and as a treatment for inflammation of skeletal joints (U.S. Patent No. 4,801,619 to Linblad).

The hyaluronic acid molecule is a highly charged polyanion composed of alternating units of D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose (see Meyer, K., *Fed. Proc.* 17:1075 (1958)); and Laurent, T.C., in: *Chemistry and Molecular Biology of the Intercellular Matrix II*. E.A. Balazs, ed. Academic Press, London and New York, pp. 703-712, (1970). These unbranched, elongated polysaccharide chains

can be chemically crosslinked to produce sheets or microfilms of a water-insoluble, hydrophilic sodium hyaluronate membrane which maintains the large negative electrostatic potential and permeability of the uncrosslinked molecules.

5 The preferred form of hyaluronic acid in the present invention is the sodium salt of hyaluronic acid. It is to be understood that other soluble salt forms or the acid form of hyaluronic acid can be used to practice the present invention. It is not considered critical in the present invention which salt
10 form is used. Salts that could be used include, but are not limited to, sodium potassium, ammonium and lithium.

 The preferred molecular weight fraction of hyaluronic acid encompasses polysaccharide chains composed of a repeating disaccharide unit consisting of sodium
15 glucuronate and N-acetylglucosamine, linked by a $\beta 1 \rightarrow 3$ glycosidic bond. The disaccharide units are linked by $\beta 1 \rightarrow 4$ glycosidic bonds, to form linear chains of a defined average length. These polymer chains form random coils in solution, characterized by an extensive hydration volume. In aqueous
20 solution at physiological pH and ion strength, these solutions are highly viscous with viscoelastic properties.

 The hyaluronic acid fraction used in the present invention is produced from the combs of freshly slaughtered
25 cocks. It is to be understood that other sources of hyaluronic acid can be used to obtain the preferred molecular weight fraction of hyaluronic acid. The present production process consists of solvent extraction, followed by enzymatic digestion of contaminating protein. The resulting material is purified by molecular filtration and specific fractionation, followed by
30 precipitation, washing and isolation of the active substance as the sodium salt. (See Examples I through IV)

 Other sources of hyaluronic acid include, but are not limited to, microorganisms, umbilical cords and synthesized molecules.

The preferred molecular weight of the hyaluronic acid contained in the culture and freezing medium of the present invention is between approximately 1.0×10^4 and 3×10^6 Daltons. The more preferred molecular weight of the hyaluronic acid is between approximately 5×10^5 to 1.2×10^6 Daltons. The most preferred molecular weight of the hyaluronic acid as used in the present invention is 7.5×10^5 Daltons.

The concentration of hyaluronic acid in the culture and freezing medium of the present invention is between approximately .01 and 10 mg/ml. A preferred concentration of hyaluronic acid is between approximately 0.05 and 5 mg/ml with a more preferred concentration of hyaluronic acid in the medium between approximately 0.5 mg/ml to 2 mg/ml. A most preferred concentration is approximately 1.0 mg/ml.

There are a variety of culture media that can be used in practicing the present invention. One of the most common media for the collection and freezing of embryos is Dulbecco's phosphate buffered saline (D-PBS) solution incorporating 1% to 10% fetal calf serum, new born calf serum or steer serum. If the embryos are to be kept in an embryo culture medium for a substantial period of time, the D-PBS is normally supplemented with 10% to 20% serum. Alternatively, the embryo culture medium may consist of another known medium, Ham's F-10 containing serum or specifically bovine serum albumin. Other media include but are not limited to, BWW (Brinster's, Whitten's & Biggar's & Whittingham's medium) and normal saline. All of these media are commercially available from a variety of sources. These products are generally supplied in powder form and made up into an aqueous medium prior to receiving the embryos.

Normally, the above mentioned culture media can be used as a freezing media by adding a cryoprotectant, *e.g.*, 10% glycerol, an energy source, *e.g.*, glucose/pyruvate,

antibiotics and in some cases a surfactant. In addition, a surfactant, such as poloxamer 188, can be added to the medium to retard the sticking of cells to the culture vessel. The preferred concentration of the surfactant is between 0.05 mg/ml to 10 mg/ml with a most preferred concentration of surfactant of between 0.1 mg/ml and 1 mg/ml.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Example I

Pre-Treatment of Cock's Combs

Cock's combs are minced, homogenized, and dehydrated in several changes of anhydrous acetone until the water content of the discarded acetone measures less than 2.0%. The dehydrated homogenate is vacuum dried to a powder. The powder is digested enzymatically with papain in a buffered aqueous medium containing cysteine hydrochloride. Celite® is added and the resulting mixture is clarified by filtration. The clear liquid filtrate has a pH between 5.0 and 7.0, a total mucopolysaccharide concentration of between 2.0 and 6.0 mg/ml sodium hyaluronate as determined by glucuronic acid assay, and an amino acid concentration of greater than 6.0 mg/ml as determined by ninhydrin assay. The clear liquid is ultrafiltered using a membrane with a molecular exclusion limit of 3×10^4 Daltons. The residual clear liquid is concentrated by ultrafiltration and the ultrafiltrate is discarded.

Example II

Complexing, Fractionation, Precipitation

NaCl (to 0.1 M) and cetylpridium chloride (CPC) are added to the residual clear liquid with agitation. Celite® is added and the precipitate is collected by centrifugation and washed three times in 0.01 M NaCl with 0.05% CPC. The precipitate is then dispersed in 0.05 M NaCl with 0.05% CPC, agitated and centrifuged. The cloudy supernatant is discarded. This procedure is repeated several times using 0.1 M NaCl with 0.05% CPC. The precipitate is then extracted in 0.3 M NaCl with 0.05% CPC, by agitation and centrifuged. Both the precipitate and the clear supernatant are saved. The extraction step is repeated three times, the final precipitate is discarded, and the clear supernatants are pooled. The pooled supernatants are brought to 0.23 M NaCl, CPC is added, the mixture is poured through Celite® packs, and filtered. The filtrate has a sodium hyaluronate concentration between 2.5 to 5.0 mg/ml as determined by glucuronic acid assay. The filtrate is then ultrafiltered using a membrane with a molecular exclusion limit of 3×10^4 Daltons. The residual liquid is concentrated by ultrafiltration and the ultrafiltrate is discarded.

Example III

Isolation of Hyaluronic Acid

The concentrated residual liquid is precipitated with 95% ethanol. The precipitate is collected by centrifugation and the supernatant is discarded. The precipitate is dissolved in 0.1 M NaCl, precipitated again with 95% ethanol, and washed. The washed precipitate contains a crude product (hyaluronic acid) of average molecular weight 2.5 to 3.5×10^5 Daltons. The yield is equivalent to 0.6% of original fresh tissue.

Example IV
Purification of Hyaluronic Acid Fraction

5 The precipitate is dissolved in pyrogen-free
distilled water (10 mg/ml). The solution is ultrafiltered using
a membrane with a molecular exclusion limit of 2×10^5
Daltons. The residual fluid is concentrated to 10% of its
original volume by ultrafiltration without addition of
10 supplementary water and the ultrafiltrate is discarded. The
concentrated residual liquid is diluted with two volumes of
pyrogen-free distilled water and the ultrafiltration and
concentration steps are repeated. This operation is repeated
two additional times. The concentrated residual liquid is
15 diluted with pyrogen-free distilled water to a concentration of
5 mg hyaluronic acid/ml. The solution is brought to 0.1 M in
NaCl and the solution is precipitated with four volumes of
95% ethanol. The precipitate is washed and then vacuum
dried. The yield of sodium hyaluronate yield equal to 0.2% of
the original tissue.

20

Example V

25 A freezing medium is prepared in Dulbecco's
phosphate-buffered saline (Gibco Laboratories, Inc.,
Burlington Ontario) supplemented with glucose-pyruvate,
antibiotics, 10% glycerol and 0.1% hyaluronic acid (1 mg/ml)
or 20% newborn calf serum as indicated. The medium has the
following formula:

5	Dulbecco's PBS	
	Na hyaluronate or Newborn calf serum	1 mg/ml 20%
10	Glucose	1 g/L
	Na pyruvate	40 mg/L
	Sodium penicillin	100,000 units/L
	Streptomycin sulfate	100,000 µg/L
	Amphotericine B	250 µg/L
	Glycerol	10% wt/vol.

15 Mouse morulae are collected on day 2.5 (vaginal plug = day 0) from CDI 3-week old mice. The embryos are washed three times in a phosphate-buffered saline solution supplemented with hyaluronic acid or newborn calf serum as indicated. Embryos are equilibrated at room temperature for ten minutes in the freezing medium with hyaluronic acid or with newborn calf serum as indicated, then loaded onto 0.25 ml straws. The straws are seeded at -7°C and cooled at 20 0.5°C/min. and submerged into liquid nitrogen at -35°C.

25 After two weeks, the embryos are then thawed in a 22°C water-bath with mild agitation for one minute. Glycerol is diluted in a single step with 1.0M sucrose for ten minutes. After three washes in phosphate-buffered saline, the mouse embryos are placed in a modified Brinster's, Whitten's and Biggar's and Whittingham's culture medium (Gibco Laboratories, Burlington, Ontario) containing 0.4% bovine serum albumin, under paraffin oil and in a humidified 30 atmosphere of 5% CO₂ in air at 37°C.

After 48 hours in culture, 84 of the 106 embryos (79.2%) frozen in the medium containing hyaluronic acid developed to expanded or hatched blastocysts (see Table I).

Example VI

5 A freezing medium is prepared in Dulbecco's phosphate-buffered saline culture medium containing glucose-pyruvate, antibiotics, 10% glycerol and 0.1% hyaluronic acid (1 mg/ml) or 20% newborn calf serum as indicated. A total of fifty-nine seven day bovine embryos (morulae to blastocyst) are collected and washed seven times in phosphate-buffered solution with hyaluronic acid or newborn calf serum as indicated. The bovine embryos are equilibrated in the freezing medium at room temperature for ten minutes and then loaded onto 0.25 ml straws. The straws are seeded at -7°C, cooled at 0.5°C/min. and then plunged at -35°C into liquid nitrogen.

15 After two weeks the embryos are then thawed at 22°C in air for five minutes. Glycerol is diluted in a single step with 1.0M sucrose for ten minutes. The embryos are washed three times in phosphate-buffered solution, then placed in Ham's F-10 (Gibco Laboratories, Burlington, Ontario) culture medium containing 0.4% bovine serum albumin under paraffin oil and in a humidified atmosphere of 5% CO₂ in air at 37°C.

20 After forty-eight hours in culture, 67.7% of the bovine embryos frozen in the medium containing hyaluronic acid developed to expanded or hatched blastocysts.

25 Table 1 summarizes the results of the two experiments described in Examples I and II in which mouse and bovine embryos are frozen in a medium containing hyaluronic acid or newborn calf serum as indicated.

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Table 1

	Total No. of Embryos	No. Embryos developed	% Survival rate
Cattle/NCS	61	41	67.2
Cattle/HA	59	40	67.7
Mouse/NCS	101	85	84.1
Mouse/HA	106	84	79.2

As can be seen in Table 1, the freezing medium containing hyaluronic acid is as effective in maintaining viability of the embryos as the freezing medium containing newborn calf serum.

Example VII

A determination is made of which molecular weight hyaluronic acid is most effective in terms of survival rate of the mouse embryos. The details of this experiment are set forth below.

The freezing medium described in Example 1 is prepared using hyaluronic acid of three different molecular weights to determine if the molecular weight of hyaluronic acid would affect embryo survival rates. Seventy-six embryos are frozen in a freezing medium containing hyaluronic acid (HA1) with a molecular weight of less than 3×10^5 Daltons (molecular weight of the hyaluronic acid varies from approximately 3×10^4 to approximately 2.5×10^5 Daltons). Fifty-eight embryos are frozen in a freezing medium with hyaluronic acid of a molecular weight of approximately between 5×10^5 and 7.5×10^5 Daltons (HA2). Sixty-seven embryos are frozen in a freezing medium with hyaluronic acid of a molecular weight approximately greater than 1.2×10^6 Daltons (HA3).

The embryos are frozen and thawed by the same method described in Example 1. Table 2 shows the survival rates for the embryos.

Table 2

	Total No. of Embryos	No. Embryos Developed	% Survival Rate
HA1	76	14	18.6
HA2	58	51	87.9
HA3	67	62	92.5

5 As shown in Table 2, the hyaluronic acid that has a molecular weight greater than 500,000 Daltons produced a survival rate greater than 87.9% while the hyaluronic acid that has a molecular weight less than 300,000 Daltons has a survival rate of only 18.6%.

10 It should be understood, of course, that the foregoing relates only to a preferred embodiment of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

What is Claimed is:

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1. A composition for culturing and freezing cells or tissues comprising a solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products.

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2. The composition of Claim 1, wherein the hyaluronic acid has a molecular weight of between approximately 1.0×10^4 to 3×10^6 Daltons.

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3. The composition of Claim 1, wherein the hyaluronic acid has a molecular weight of between approximately 5×10^5 to 1.2×10^6 Daltons.

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4. The composition of Claim 1, wherein the hyaluronic acid has a molecular weight of approximately 7.5×10^5 Daltons.

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5. The composition of Claim 1, wherein the cells are embryos.

6. The composition of Claim 1, wherein the cells are unfertilized ova.

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7. The composition of Claim 1, wherein the cells are sperm.

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8. The composition of Claim 1, wherein the medium further comprises an effective amount of surfactant.

9. The composition of Claim 8, wherein the surfactant is poloxamer 188.

5 10. A method for culturing cells or tissues comprising the step of mixing the cells or tissues with a medium, the medium comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products.

10 11. The method of Claim 10, wherein the hyaluronic acid has a molecular weight of between approximately 1.0×10^4 to 3×10^6 Daltons.

15 12. The method of Claim 10, wherein the hyaluronic acid has a molecular weight of between approximately 5×10^5 to 1.2×10^6 Daltons.

20 13. The method of Claim 10, wherein the hyaluronic acid has a molecular weight of approximately 7.5×10^5 Daltons.

25 14. The method of Claim 10, wherein the cells are embryos.

15. The method of Claim 10, wherein the cells are unfertilized ova.

30 16. The method of Claim 10, wherein the cells are sperm.

17. The method of Claim 10, wherein the medium further comprises an effective amount of surfactant.

18. The method of Claim 17, wherein the surfactant is poloxamer 188.

19. A method for freezing cells or tissues comprising the steps of

- a. mixing the cells or tissues with a medium, the medium comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products; and
- b. lowering the temperature of the culture medium sufficiently to preserve the embryos in a viable condition.

20. The method of Claim 19, wherein the hyaluronic acid has a molecular weight of between approximately 1.0×10^4 to 3×10^6 Daltons.

21. The method of Claim 19, wherein the hyaluronic acid has a molecular weight of between approximately 5×10^5 to 1.2×10^6 Daltons.

22. The method of Claim 19, wherein the hyaluronic acid has a molecular weight of approximately 7.5×10^5 Daltons.

23. The method of Claim 19, wherein the cells are embryos.

24. The method of Claim 19, wherein the cells are unfertilized ova.

25. The method of Claim 19, wherein the cells are sperm.

26. The method of Claim 19, wherein the medium further comprises an effective amount of surfactant.

5 27. The method of Claim 26, wherein the surfactant is poloxamer 188.

10 28. A method for flushing ova or embryos from a donor female comprising the step of flushing the reproductive tract of an appropriate female with a medium, the medium comprising a culture solution containing the nutritional substances necessary to support the cells or tissues and hyaluronic acid in a concentration effective to be a substitute for serum or serum products.

15 29. The method of Claim 28, wherein the hyaluronic acid has a molecular weight of between approximately 1.0×10^4 to 3×10^6 Daltons.

20 30. The method of Claim 28, wherein the hyaluronic acid has a molecular weight of between approximately 5×10^5 to 1.2×10^6 Daltons.

25 31. The method of Claim 28, wherein the hyaluronic acid has a molecular weight of approximately 7.5×10^5 Daltons.

30 32. The method of Claim 28, wherein the medium further comprises an effective amount of surfactant.

 33. The method of Claim 32 wherein the surfactant is poloxamer 188.

INTERNATIONAL SEARCH REPORT

PCT/CA 91/00186

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A01N1/02; C12N5/00; //A61K35/54; A61K35/52 A61D19/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K ; A01N ; C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	THERIOGENOLOGY vol. 35, no. 1, January 1991, PALO ALTO, CA, US page 251; A. PALASZ ET AL.: 'THE USE OF HYALURONIC ACID AS A MACROMOLECULE IN MOUSE EMBRYO VITRIFICATION MEDIA' see the whole document ---	1-33
X	THERIOGENOLOGY vol. 33, no. 1, January 1990, PALO ALTO, CA, US page 295; A. PALASZ ET AL.: 'SUBSTITUTION OF CHEMICALLY DEFINED MACROMOLECULES FOR PROTEIN IN MURINE EMBRYO FREEZING MEDIUM' see the whole document --- -/-	1-33
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
10 FEBRUARY 1992	13.02.92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O. <i>Ant. S. Ryckebosch</i>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>WORLD PATENTS INDEX Section Ch, Week C10, 16 April 1980 Derwent Publications Ltd., London, GB; Class C, AN 80-17848C & SU,A,670 301 (AGRIC LIVESTOCK BRE) 30 June 1979 see abstract</p> <p>---</p>	1,7,10, 16,19,25
A	<p>CHEMICAL ABSTRACTS, vol. 104, no. 14, 7 April 1986, Columbus, Ohio, US; abstract no. 116062, Y. IMAI ET AL.: 'EFFECT OF MOBILE MOLECULAR CHAINS ON CELL ATTACHMENT AND GROWTH.' page 398 ; see abstract</p> <p>---</p>	1,8-10, 17-19, 26-28, 32,33

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9100186
SA 47783

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 10/02/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
/*- - ----- <div style="text-align: center; margin-top: 10px;">None</div>			

EPO FORM P0079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82